ANOVULATORY FOLLICLE DEVELOPMENT IN ADULT ALBINO MICE DUE TO PROLONGED EXPOSURES TO GENISTEIN

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ABSTRACT: Genistein is a soy phytoestrogen that mediates its functions by interacting with the endogenous estrogen receptor β. To investigate the effect of 10mg/kg body weight/day genistein administration up to 90 days on the ovarian folliculogenesis and its correlation with serum gonadotropins and estrogen, adult Mus musculus were either administered with 1:4 DMSO : PBS (vehicle) or with 10mg genistein/kg body weight dissolved in vehicle for 30, 60 and 90 days. Post sacrifice on 31st, 61st and 91st day, blood serum was separated and the gonadotropins (LH and FSH) and estrogen hormone was estimated by ELISA and Bouin’s fixed ovaries of control and treated mice were used for histology after H&E staining. Comparison of the ovarian sections revealed increased number of arrested anovulatory follicles along the treatment groups i.e., 30, 60 and 90 days and chronological decrease in the serum concentrations of LH, FSH as well as estrogens (two way ANOVA, at p<0.05 to p<0.001). Together these results suggested for delayed folliculogenesis and reduced number of mature oocytes, which could be correlated with less to nil offspring production signifying lesser fertility or sometimes severity may end up in sterility.

INTRODUCTION: It is well documented that during the normal process of ovarian differentiation, the germ cells form clusters or nests, which have similarities with the germ line cysts found in female invertebrates and males of almost all species of animals 1, 2. These cysts are formed as a result of series of incomplete cell divisions of the oogonia just after their formation. These cells remain connected with each other and subsequently undergo meiotic divisions where after they are called oocytes 1. These germ-line cysts or oocyte nests of mouse dissociate post partum and become surrounded with pre-granulosa cells to form individual primordial follicles 3. The dissociation of the germline cysts are known as nest breakdown, during which 2/3rd of the total number of oocytes disintegrates and only 1/3rd of them survive 3. These events eventually give rise to two sub-populations of oocytes-one being those which survive, differentiate and form the functional oocytes and the other that support and protect the oocytes but eventually die during the process. Estrogens have been reported to be involved in the...
regulation of the programmed cell death and survival of the oocyte sub-populations. It is reported that FSH inhibits follicular atresia and higher concentration of estrogen inhibits secretion of FSH and is also responsible for atresia. However, estrogens have been shown to protect granulosa cells from undergoing atresia. In contrast to these there are evidences that estrogens promote cell death in the organ systems like nervous system. Whether estrogen treatment regulates the number of degenerative oocytes during nest breakdown is not well established.

Over the years estrogen and their analogues have been shown to induce various effects on the reproductive system like abnormality in tissue morphology as well as neoplasia. Most conspicuous of them is the formation of multi-oocyte follicles (MOFs) in the ovaries of adult animals. Neonates treated with either endogenous estradiol 17β (E2) or its synthetic analogues (like Diethylstilbestrol/DES) show increased incidence of developing MOFs during adulthood as compared to the normal ovaries. In spite of these reports the origin of MOFs still remains a mystery. There are many plausible explanations available like, treatment with E2 during neonatal ovarian differentiation results in the formation of oocyte nests that persists and do not undergo breakdown. Later on these nests become surrounded by granulosa cells and results in the formation of follicles with more than one oocyte (MOFs) in adult ovary. Another possible argument may be that, nest breakdown may occur but migration of the granulosa cells around the oocyte may get affected. It may also be the case where two or more follicles may fuse together to form one follicle. In argument if estrogen is the causative factor behind the formation of MOFs, then chemicals with estrogenic properties should also entail for similar kind of effects.

Genistein is a soy derived isoflavonoid compound shown to possess estrogenic activities both in vitro as well as in vivo through its ability to bind to the estrogen receptor β, hence known as phytoestrogen. Numerous studies have been carried out on mouse and rat models that evidences for the adverse reproductive outcomes of genistein on female systems post administration. Some of these soy-based edible products have been reported to have high concentrations of genistein. Soy-based infant formulations consume approximately 6–9 mg/kg per day of genistein compared to 1 mg/kg per day in an adult vegetarian diet. Studies also report that neonatal exposure to genistein at a dose of 50mg/kg body weight/day (during post natal day 1-5) leads to an increased incidence of uterine adenocarcinoma (35%) during adulthood; in comparison to 0.001mg/kg body weight/day DES treated animals (31%) Studies have reported that exposure to genistein during prenatal developmental stages may cause altered reproductive cyclicity, ovarian function as well as subfertility and infertility in adult rodents. However, sufficient number of studies has not been carried out regarding effects of genistein administration in adult mice. Since soy and soy based edible products and formulations are most widely used in preparations of the vegetarian style diets in India. The study aims to determine the effects of genistein at a dose of 10mg/kg body weight/day (dissolved in 1:4 DMSO: PBS) upto 90 days, on the histomorphology of the ovary and also to correlate the alterations produced with the changes in the circulating levels of gonadotropins (LH and FSH).

**MATERIALS AND METHODS:**

Approximately 7-8 week old thirty female albino mice were housed and acclimated at approximately 24 ± 2 °C temperature with 10h : 14h light : dark cycle in the animal house of the Laboratory of Endocrinology, at the Department of Bioscience, Barkatullah University, Bhopal, Madhya Pradesh, India following the guidelines of the Institutional Animal Ethics Committee (IAEC) formed under CPCSEA, Govt. Of India.. They were provided commercially available mice feed and water ad libitum.

Commercially available 98% pure HPLC grade Genistein powder purchased from Sigma Aldrich was used to prepare a dose containing 10 mg Genistein / kg body weight / day, dissolved in 1 : 4 Dimethyl sulfoxide (DMSO) : Phosphate buffered saline (PBS).

Fifteen females were administered orally 10 mg Genistein/kg body weight/day, dissolved in 1 : 4 Dimethyl sulfoxide (DMSO):Phosphate buffered saline (PBS) up to 90 days and remaining fifteen received equal volume of vehicle (1: 4, DMSO : PBS) alone for 90 days.

On 31st, 61st and 91st day five mice were sacrificed by cervical dislocation and their blood were collected into sterile collection vials by cardiac...
puncture method. Sera were separated by appropriate technique and estimation of Luteinizing hormone (LH), Follicle stimulating hormone (FSH) and Estrogen (E2) hormone were done using appropriate ELISA assay kits developed by My Bio-Source, Inc., San Diego, USA. Two-way ANOVA analyses were performed to compare and assess any at all differences (at p < 0.05 to p < 0.001) in the serum LH, FSH and E2 hormone concentrations obtained from the control and experimental groups after different intervals. Bouin’s fixed ovaries of control and genistein treated animals were used to prepare 5-7 μ (micron) sections and stained following standard H&E method. Photomicrography was done at 100X and 400X magnifications with the help of a photomicrography unit (Motic-DMB1-B microscope, fitted with digital camera).

RESULTS:
The results show that there had been chronological decrease in the LH, FSH as well as Estrogen concentrations. Concentrations of LH decreased significantly (p<0.01, via two way ANOVA analysis) after 30, 60 and 90 days of genistein administration (Figure 1).

The decrease in serum FSH concentration was not significant after 30 days of treatment but significantly decreased (p<0.05, via two way ANOVA analysis) sequentially after 60 and 90 days genistein treatment (Figure 2). There was significant (p<0.05, via two way ANOVA analysis) chronological decrease in the circulatory level of estrogen in comparison to the vehicle treated control animals (Figure 3).

FIGURE 1: COMPARISON OF CIRCULATING LUTEINIZING HORMONE (LH) LEVEL (MIU/ML), WITHIN CONTROL AND GENISTEIN TREATED FEMALE MUS MUSCULUS, AFTER 30, 60 AND 90 DAYS

FIGURE 2: COMPARISON OF CIRCULATING FOLLICLE STIMULATING HORMONE (FSH) LEVEL (MIU/ML), WITHIN CONTROL AND GENISTEIN TREATED FEMALE MUS MUSCULUS, AFTER 30, 60 AND 90 DAYS

FIGURE 3: COMPARISON OF CIRCULATING ESTROGEN HORMONE (E2) LEVEL (NG/ML), WITHIN CONTROL AND GENISTEIN TREATED FEMALE MUS MUSCULUS, AFTER 30, 60 AND 90 DAYS

FIGURE 4: LONGITUDINAL SECTION OF CONTROL AND 30, 60 AND 90 DAYS GENISTEIN TREATED Ovary OF MICE, MUS MUSCULUS
Histopathological observations of the H-E stained ovaries under 100X as well as 400X magnifications revealed chronological increase in the number of follicles along the 30, 60 and 90 days genistein treated animals. H-E stained longitudinal sections (LS) of the control ovary shows different follicular developmental stages scattered through the medullar region of the ovary. The mature follicles lined around the periphery. The germinal epithelium (GE) is properly organized. Primary as well as secondary follicles were prominent. The oocyte is located in a mound of cumulus oophorus (CO) or granulosa cells that are normal in appearance. One developing antrum/follicular cavity is visible. The membrana granulosa (MG) is also persistent (Figure 4: A & B).

Figure 4: C and D depict the ovarian histology of 30 days genistein treated female. The LS shows the cortex as well as the medullar region that are filled with lesser number of developing follicles but more atretic follicles. Proper MG is seen. The follicles are filled with granulosa cells and oocytes are missing. Numerous GC filled follicles are visible. One primary follicle is seen but that is within the periphery of the two follicles.

Figure 4: E and F show the ovarian histology after 60 days of genistein administration to female mice. Growing number of follicles filled with GC and less Number of mature follicles are evident. Some arrested follicles at their secondary stage of maturation are seen. Proper MG and GC are evident.

Figure 4: G and H show the ovarian histology after 90 days of genistein administration to female mice. Closely packed anovulatory/arrested follicles filled with GC are seen. Distorted GE is seen. Although primary follicles are present but their numbers are few. Large GC filled follicles are evident.

DISCUSSIONS: In the current study, we tried to evaluate the effects of genistein exposure on the mature mouse ovary and the circulating levels of the gonadotropins (LH & FSH) and estrogen hormone. Several studies done earlier have shown that neonatal exposure to the soy phytoestrogen genistein alters ovarian morphology later in life, including the presence of MOFs 27. There may be several possible ways by which such abnormalities may occur. Like, the granulosa cells could improperly enclose more than one oocyte, disruption in the process of oocyte nest breakdown or, fusion of more than two follicles. Although various reports of MOF formation during neonatal genistein exposure have been reported, there are no such reports on the adult mature ovary.

Development of the ovarian follicles is a complex process involving endocrine, paracrine as well as autocrine regulations. The follicular developmental stages are primordial, secondary and pre-antral. The mature follicles there after either undergo atresia or under the control of cyclical gonadotropin stimulations they reach pre-ovulatory antral stage followed by release of ovum (ovulation) under the influence of estrogen 28. Folliculogenesis involves interactions between different ovarian cell types and these cells are triggered by the gonadotropins- FSH and LH. These hormones control the production of ovarian steroids wherein, LH stimulates the theca interna cells to produce aromatizable androgens 29, 30. The granulosa cells are induced to produce P-450 aromatase (CYP19) under the influence of FSH which then converts the androgens into estrogens in these cells 29, 30, 31.

This study shows that there had been significant drop in the levels of gonadotropins as well as estrogen. Researchers have shown that estrogens protect the granulosa cells from undergoing apoptosis, whereas the androgens enhance GC apoptosis 32. Therefore it is possible that the growing concentration of estrogen have protected the developing cells from undergoing apoptosis and therefore reduction in the number of maturing follicles and increase in atresia. The increased concentration of estrogen signals the pituitary to stop synthesis of FSH and LH therefore in turn blocks folliculogenesis. To the contrary, genistein is an estrogen mimic that also mediates its functions interacting with the estrogen receptor β, and that may have caused the increase in the number of GC filled follicles in adult ovary. Unlike neonatal treatment with genistein that causes MOF formation in the ovaries, prolonged treatment (upto 90 days) with genistein causes formation of GC filled follicles. Although less amount of endogenous estrogen may be available, the block in apoptosis may have been served by genistein.

In one study in rats, neonatal progesterone treatment have been found to reduce primordial follicle assembly in contrast to both progesterone and estrogen treatment, which reduced the
transition from primordial to primary follicle in the initial steps of folliculogenesis. However, oocyte nest breakdown was not specifically examined; this study also showed reduced neonatal oocyte apoptosis in progesterone treated mice. Together these data suggested that the steroid hormone balance may also be important for proper ovarian differentiation and development. Research on phytoestrogens, like genistein, has increased over the last decade. There are certain contradictory results that have been put forward to the scientific community regarding the beneficial as well as adverse effects of genistein in a dose and duration dependant manner. Some studies have shown that exposure to genistein during early developmental periods prevents carcinogen induced mammary gland cancer, while others have reported the occurrence of ovarian follicle atresia. 

It has been reported that genistein improves cholesterol synthesis rates of human infants consuming soy-based formulas. Vegetarian diets usually contain high levels of soy, and recent epidemiology reports have shown an association of a vegetarian diet during pregnancy and recent epidemiology reports have shown an association of a vegetarian diet during pregnancy with an increased incidence of hypospadias in the male offspring. Correlations have also been found between the increase in autoimmune disease and the use of allergy medicines in children fed soy-based infant formulas. Not only genistein, but neonatal exposure to different environmental estrogen such as bisphenol A, has also been shown to cause MOFs in mice. This finding further supports the idea that estrogenic activity of any exogenous substance may cause altered ovarian differentiations.

CONCLUSION: it can be proposed that ovarian differentiation is a complex and multifaceted process; in which, disruption in any of the pathways can lead to alterations in the normal progression of ovarian development and subsequent normal ovarian function. Therefore unlike neonatal exposure to genistein that causes early differentiation defects in folliculogenesis; genistein exposure at a dose of 10mg/kg body weight/day until 90 days, during adulthood causes reduced atresia, development of GC rich anovulatory follicles as well as block during secondary follicular growth and differentiations. Which are signs of reproductive malfunctioning in female subjects exposed to genistein.

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REFERENCES:
18. Milligan SR, Balasubramanian AV and Kalita JC: Relative potency of xenobiotic estrogens in an acute in vivo

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